

# Involvement of GSK-3 in Regulation of Murine Embryonic Stem Cell Self-Renewal Revealed by a Series of Bisindolylmaleimides

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## SUMMARY

The ability to propagate embryonic stem cells (ESCs) while maintaining their pluripotency is critical if their potential use in regenerative medicine is to be realized. The mechanisms controlling ESC self-renewal are under intense investigation, and glycogen synthase kinase 3 (GSK-3) has been implicated in regulating both self-renewal and differentiation. To clarify its role in ESCs we have used chemical genetics. We synthesized a series of bisindolylmaleimides, a subset of which inhibit GSK-3 in murine ESCs and robustly enhance self-renewal in the presence of leukemia inhibitory factor (LIF) and serum, but not in the absence of LIF. Importantly, these molecules appear selective for GSK-3 and do not perturb other signaling pathways regulating self-renewal. Our study clarifies the functional importance of GSK-3 in regulation of ESC self-renewal and provides tools for investigating its role further.

## INTRODUCTION

Embryonic stem cells (ESCs) are derived from the inner cell mass of preimplantation embryos and can differentiate into all cells comprising the three germ layers of the developing embryo (Smith, 2001). This property of pluripotency makes ESCs attractive for the generation of specific cell types for use in regenerative medicine and drug screening and as a model system of early development. In order to harness their potential, the molecular mechanisms regulating ESC self-renewal need to be understood such that pluripotency can be maintained during expansion. In the ESC context, self-renewal is essentially proliferation accompanied by the suppression of differentiation and can be defined as the symmetrical division of ESCs to produce two identical undifferentiated daughters.

A network of extrinsic factors, signaling pathways, and transcriptional regulators is involved in regulation of ESC self-renewal, underpinning maintenance of pluripotency (Boiani and Schoeler, 2005). The transcription factors Oct4, Sox2, and Nanog each play a key role in maintenance of ESC pluripotency (Boiani and Schoeler, 2005; Niwa, 2007). Of the extrinsic factors,

leukemia inhibitory factor (LIF) is important for maintaining self-renewal of murine ESCs (mESCs) (Smith et al., 1988; Smith and Hooper, 1987) via activation of STAT3 (Boeuf et al., 1997; Matsuda et al., 1999; Niwa et al., 1998; Raz et al., 1999) and induction of c-Myc (Cartwright et al., 2005). LIF also activates the Ras/Erk kinase pathway (Burdon et al., 1999; Ernst et al., 1996), ribosomal S6 kinases (Boeuf et al., 2001), phosphoinositide 3-kinases (PI3Ks) (Paling et al., 2004), and Src kinases (Anneren et al., 2004). LIF-induced ERK activation appears to promote differentiation (Burdon et al., 1999), leading to the proposal that the balance between STAT3 and ERK signals are important in determining mESC fate (Burdon et al., 2002). In the absence of serum or feeder cells, LIF is not sufficient to maintain mESC self-renewal but, under chemically defined conditions, bone morphogenetic protein 4 can synergize with LIF to maintain self-renewal via induction of *Id* (inhibitor of differentiation) genes (Ying et al., 2003) and inhibition of p38 mitogen-activated protein kinase (MAPK) signaling (Qi et al., 2004).

Activation of Wnt signaling and subsequent inhibition of GSK-3 activity has also been implicated in maintaining self-renewal of ESCs. Sato et al. used the GSK-3 inhibitor 6-bromindirubin-3-oxime (BIO; Meijer et al., 2003) as a surrogate activator of Wnt signaling and reported that BIO enhanced self-renewal of both mESCs and human ESCs (hESCs), although these effects were not quantified at a clonal level (Sato et al., 2004). Subsequently, it has been reported that conditioned media containing Wnt3a can support self-renewal of mESCs (Hao et al., 2006; Ogawa et al., 2006; Singla et al., 2006) and hESCs (Dravid et al., 2005; Sato et al., 2004). Tcf-3 was recently shown to co-occupy promoters with Nanog and Oct4, leading to the suggestion that it integrates Wnt signals with the core regulatory factors in ESCs (Cole et al., 2008). However, Wnt/ $\beta$ -catenin signaling alone is not sufficient to maintain self-renewal, as Wnt3a alone stimulates hESC differentiation and does not maintain mESC pluripotency (Dravid et al., 2005; Ogawa et al., 2006; Singla et al., 2006). In addition, expression of an activated mutant of  $\beta$ -catenin does not maintain self-renewal of mESCs, whereas overexpression can induce neuronal lineage commitment (Otero et al., 2004). Furthermore,  $\beta$ -catenin null mESCs maintain expression of pluripotency markers (Anton et al., 2007). Intriguingly, a distinct GSK-3 inhibitor (TWS119) has been reported to induce neuronal differentiation of embryonal carcinoma cells and ESCs, as opposed to self-renewal (Ding et al., 2003), seemingly at odds with a role for GSK-3 in controlling self-renewal and questioning

how GSK-3 influences ESC fate. There is further complexity, because GSK-3 is involved in many additional signaling processes (Cohen and Frame, 2001; Doble and Woodgett, 2003). Importantly, the PI3K/Akt pathway regulates GSK-3 activity in response to cytokines, and a role for PI3K/Akt signaling in regulation of self-renewal of both mESCs (Paling et al., 2004; Watanabe et al., 2006) and hESCs (Armstrong et al., 2006; Pyle et al., 2006) has been demonstrated.

Thus, the functional role that GSK-3 plays in ESC biology remains enigmatic, and we rationalized that an independent approach to investigating GSK-3 function in ESCs was warranted. We have shown that the conformation of macrocyclic bisindolylmaleimides can be a critical factor in their relative ability to inhibit alternative protein kinases (Bartlett et al., 2005). From these studies, compound **1i** was found to be a selective small-molecule ATP-competitive inhibitor of GSK-3 $\beta$  (Bartlett et al., 2005). We have used **1i** as a lead molecule and have synthesized a further panel of ligands, including potent and selective GSK-3 inhibitors, to investigate the role of GSK-3 in mESCs. Our results demonstrate that inhibition of GSK-3 robustly enhances mESC self-renewal in the presence of LIF and serum, but cannot sustain self-renewal in the absence of LIF. Importantly, our compounds selectively inhibit GSK-3 and do not affect signaling via Stat3 and MAPK pathways. Our results highlight a clear functional role for GSK-3 in regulation of ESC self-renewal.

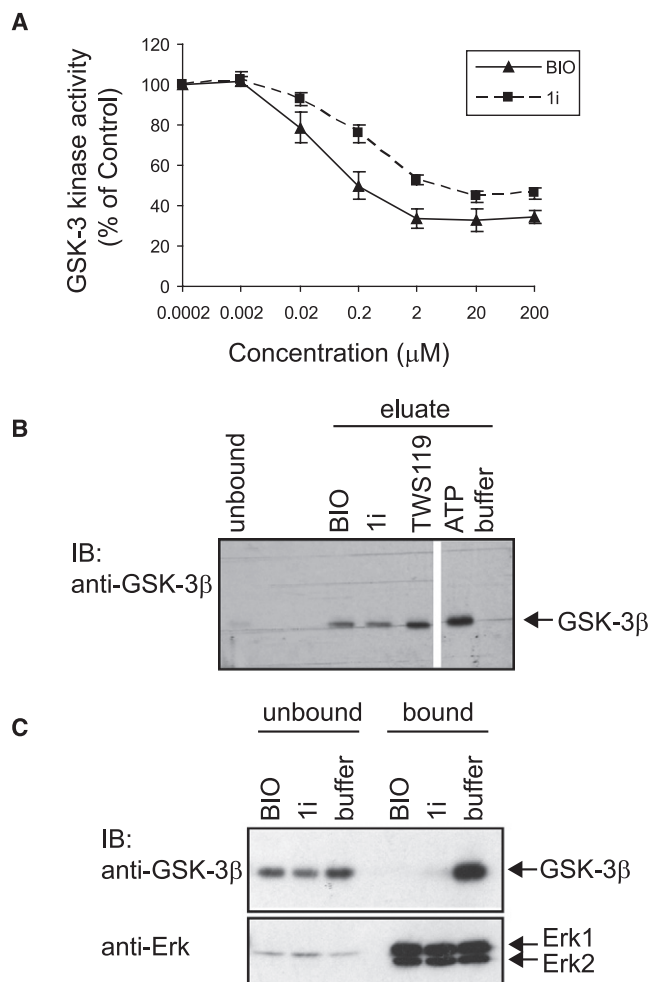
## RESULTS

### Compound **1i** Is a GSK-3 Inhibitor in ESCs

Previously, we tested a series of novel bisindolylmaleimides for activity against a panel of 29 protein kinases in an in vitro screen using ATP concentrations close to the  $K_m$  value for each individual kinase (Davies et al., 2000). In this assay, compound **1i** in our current scheme (see below) was demonstrated to have selectivity for GSK-3 $\beta$  (Bartlett et al., 2005). As a starting point, it was important to demonstrate that **1i** could target GSK-3 in ESCs, so we directly compared the ability of **1i** and BIO to inhibit GSK-3 activity in ESC lysates (Figure 1A). **1i** inhibited GSK-3 kinase activity with an  $IC_{50}$  of 250 nM but was less potent than BIO, which had an  $IC_{50}$  of 50 nM. Using recombinant GSK-3 $\beta$  protein, we obtained an  $IC_{50}$  value of 20 nM for **1i** (see Table 1; at an ATP concentration of 10  $\mu$ M), compared to a published  $IC_{50}$  value of 5 nM for BIO (Meijer et al., 2003), suggesting an approximately 5-fold difference in the ability of **1i** to inhibit GSK-3 activity compared to BIO.

Affinity purification demonstrated an interaction of **1i** with GSK-3 in ESC extracts. KinaseBind  $\gamma$ -phosphate-linked ATP agarose was used as an affinity matrix to enrich ATP-binding proteins from ESC lysates. As seen in Figure 1B, BIO, **1i**, TWS119, and ATP all eluted GSK-3 $\beta$  from the ATP agarose. In addition, preincubation of ESC lysates with BIO or **1i**, prior to incubation with the ATP agarose, abolished binding of GSK-3 $\beta$  but not of the unrelated kinase Erk1 (Figure 1C). These data confirm that BIO and **1i** compete with the ATP binding site of GSK-3 $\beta$  in ESCs, consistent with previous reports (Bartlett et al., 2005; Meijer et al., 2003).

**1i** has previously been shown to be selective for GSK-3 $\beta$  (Bartlett et al., 2005). To further explore **1i** selectivity, we



**Figure 1. Compound **1i** Inhibits GSK-3 Activity in ESC Lysates**

(A) ESC lysates were incubated with different concentrations of BIO (filled triangles) or **1i** (filled squares) in the presence of [ $\gamma$ - $^{32}$ P]ATP and substrate peptide for 15 min. Data (mean  $\pm$  SEM) from three to five independent experiments are expressed as percentage of GSK-3 activity in the absence of inhibitor (% control). Incomplete inhibition was observed, likely to be related to nonspecific background, also noted in GSK-3 $\alpha/\beta$  null ESCs (Doble et al., 2007). (B) Elution assay: KinaseBind  $\gamma$ -linked ATP agarose beads were incubated with ESC lysates. A sample of the extract was saved (unbound), beads were washed, and bound protein was eluted with 50  $\mu$ M indicated compound (or 10  $\mu$ M ATP). The eluates were fractionated by SDS-PAGE on the same 10% acrylamide gel and immunoblotted with anti-GSK-3 $\beta$  antibodies. The space between samples is due to removal of two intervening lanes not required for the figure. (C) Competition assay: ESC extracts were preincubated with 5  $\mu$ M BIO or **1i** prior to incubation with KinaseBind  $\gamma$ -linked ATP agarose. Samples of extract not bound to the agarose were taken (unbound) and the beads were washed. The agarose was then boiled in sample buffer to extract the bound proteins (bound). The samples were immunoblotted with anti-GSK-3 $\beta$  antibodies or anti-Erk antibodies as indicated.

analyzed changes in protein phosphorylation and expression using the Kinex antibody microarray (Kinexus, Vancouver, BC, Canada), comprising 250 phospho-site and 350 pan-specific antibodies. Following 1 or 24 hr treatment with 5  $\mu$ M **1i**, phosphorylation of very few proteins were significantly altered compared to controls (see Table S1 available online), indicating **1i** does not have significant off-target effects.

### Compound **1i** Enhances Self-Renewal of ESCs

Inhibition of GSK-3 with BIO has been reported to enhance self-renewal of mouse and human ESCs (Sato et al., 2004). Conversely, a structurally distinct GSK-3 inhibitor, TWS119, promotes neuronal differentiation of mESCs (Ding et al., 2003). We therefore determined the ability of **1i**, in direct comparison with BIO and TWS119, to enhance ESC self-renewal using clonal assays (in the presence of LIF and serum). Five days after plating, colonies were stained for alkaline phosphatase activity. Alkaline phosphatase is expressed only by undifferentiated ESCs, and stained colonies fall into two categories: (1) pure ESC colonies, which have a compact round morphology and stain intensely, indicative of a highly self-renewing state (Pure), and (2) less compact colonies with staining concentrated in the colony center (self-renewing). Treatment with 5  $\mu$ M **1i**, 0.5  $\mu$ M BIO, or 2  $\mu$ M TWS119 resulted in dramatic increases in the number of pure, highly self-renewing colonies (Figure 2A). However, above 7.5  $\mu$ M, toxicity to **1i** was observed. Following treatment with **1i**, BIO, or TWS119, expression of pluripotency markers Nanog and Oct4 were maintained, and in some cases modestly elevated (Figure 2B; Figure S1), consistent with retention of an undifferentiated phenotype.

GSK-3 plays a pivotal role in the canonical Wnt signaling pathway, and although involvement of the Wnt pathway in ESC self-renewal is controversial, measurement of Wnt signaling provides a convenient means to assess GSK-3 inhibition in ESCs. In the absence of Wnts, GSK-3 is active and phosphorylates  $\beta$ -catenin, targeting it for degradation. In the presence of Wnts, GSK-3 is inactivated,  $\beta$ -catenin phosphorylation falls allowing its levels to rise, whereupon it translocates to the nucleus and activates transcription via TCF/LEF (T cell factor/lymphoid enhancer-binding factor) transcription factors. We assessed the ability of **1i**, in comparison with BIO and TWS119, to inhibit GSK-3 activity in intact ESCs by monitoring their ability to cause a decrease in levels of  $\beta$ -catenin phosphorylation. As seen in Figure 2C, levels of  $\beta$ -catenin phosphorylation were modestly reduced with 5–7.5  $\mu$ M **1i**, whereas only 0.5  $\mu$ M BIO was required to achieve a similar decrease. In contrast, even at 10  $\mu$ M, TWS119 only led to a modest reduction in  $\beta$ -catenin phosphorylation.

To further assess the ability of **1i** to inhibit GSK-3 activity within ESCs, we used the TOPFlash luciferase reporter assay to assess upregulation of  $\beta$ -catenin-mediated TCF/LEF transcriptional activity. Treatment with 5  $\mu$ M **1i** resulted in a 5-fold increase in TCF reporter activity, comparable to the increase observed following treatment with 0.5  $\mu$ M BIO (Figure 2D). Thus, at concentrations that decrease  $\beta$ -catenin phosphorylation, both BIO and **1i** induce a corresponding 3- to 5-fold increase in TCF activity. TWS119 at 2  $\mu$ M, a dose which enhances mESC self-renewal, led only to a very modest 1.4-fold increase in TCF activity, raising the question of whether GSK-3 is the primary target for this molecule in mESCs. Together, these data indicate that **1i** and BIO enhance self-renewal of mESCs in the presence of serum and LIF, a property that correlates with their ability to inhibit GSK-3 activity.

### Effects of Related Bisindolylmaleimides on ESC Self-Renewal

To further probe the relationship between GSK-3 and ESC self-renewal, we synthesized and functionally characterized a series of 48 additional molecules related to **1i** (see Figure 3) including bisindolylmaleimides (**1**, **2**, **3**, and **6**), 7-azaindolyl indolylmaleimides

(**4** and **7**), and bis-7-azaindolylmaleimides (**5**); these derivatives were prepared by alkylation of the heterocyclic ring system and/or deprotection of the imide nitrogen atom (see Supplemental Experimental Procedures). The conformation of bisindolylmaleimide derivatives plays a major role in their ability to inhibit protein kinases (Bartlett et al., 2005). To vary the torsion angles between the maleimide and (aza)indolyl ring systems, we prepared a range of macrocyclic derivatives in which the length of the tether was varied, and some analogs in which the 2-position of an indole ring was methylated (Bartlett et al., 2005; Kuo et al., 2003). Figure 3 and Table 1 summarize the molecular structures of the compounds synthesized and the results of functional evaluation. Sixteen of the derivatives prepared were found to enhance self-renewal, 12 with potency equal or better than our lead compound **1i**.

Most of the compounds that enhanced self-renewal were macrocyclic analogs in which the (aza)indolyl nitrogen atoms were linked by a tether (Table 1). Tether length and substitution significantly affected the ability of compounds to enhance self-renewal. Changing tether length from 9 methylene groups (as in **1i**) to 8 (**1h**; Bartlett et al., 2005) or 11 (**1k**) reduced potency, whereas decreasing the tether length to 7 (**1g**) resulted in a loss of functional activity (Table 1A). However, the bisindolylmaleimides **1l** and **1m**, having polyoxygenated tethers (11 and 14 atoms, respectively), were more potent than **1i** and robustly enhanced ESC self-renewal. However, the corresponding 7-azaindolyl indolylmaleimides (**4l** and **4m**) and bis-7-azaindolylmaleimide (**5m**) derivatives were less potent (Table 1B). Interestingly, the 7-azaindolyl indolylmaleimides (**4i**) and the bis-7-azaindolylmaleimides (**5i**) had similar potencies to the corresponding bisindolylmaleimides (**1i**) (Table 1B).

The addition of a methyl group to the 2-position of an indole ring can change the ligand conformation by widening the torsion angle between that ring and the maleimide. The addition of a methyl group to one of the indoles of **1i** (to generate **2i**) slightly reduced activity, whereas the same modification of **1m** (to generate **2m**) resulted in loss of activity (Table 1A). Adding one or two methyl groups to **1i** (to give **2i** and **3i**) or to **1h** (to give **2h** and **3h**) resulted in compounds unable to enhance self-renewal. The bisindolylmaleimide **2c**, with one 2-methylindole ring, was the only untethered compound that enhanced ESC self-renewal.

A series of analogs in which the tether of **1i** was substituted were prepared (Figure 3B; Table 1C). The introduction of a hydroxyl group on the central methylene of the tether gave **1o**, a molecule that robustly enhanced self-renewal with greater potency than **1i** (the hydroxyl group may allow **1o** to form an additional H bond with its target). The addition of an allyloxy or but-3-enyl substituent at various positions gave molecules that retained similar properties to **1i** (**1p** and **1r**) or were less effective (**1q**, **1s**, and **1t**). These analyses enabled us to group our molecules based on their functional activity in ESC self-renewal. We subsequently performed more detailed analyses to investigate specific dose-response characteristics and to determine whether the potency of GSK-3 inhibition correlated with effects on self-renewal.

### Potency of GSK-3 Inhibition Correlates with the Ability to Enhance ESC Self-Renewal in the Presence of LIF and Serum

In vitro assays, using recombinant GSK-3 $\beta$ , demonstrated that three molecules, **1l**, **1m**, and **1o**, had IC<sub>50</sub> values for inhibition

**Table 1. Functional Evaluation of Bisindolylmaleimides**(A) Effects of Bisindolylmaleimides (1, 2, 3, and 6) on Self-Renewal<sup>a</sup>

Compound	R <sup>1</sup> , R <sup>2</sup>	SR <sup>b</sup>	Toxicity <sup>c</sup>	GSK-3β IC <sub>50</sub> <sup>d</sup>
1a	H,H	No	No (5 μM)	ND
1c	Me,Me	No	2 μM	ND
1e	SEM,SEM <sup>e</sup>	No	No (5 μM)	ND
1g	(CH <sub>2</sub> ) <sub>7</sub>	No	No (10 μM)	ND
1h	(CH <sub>2</sub> ) <sub>8</sub>	10 μM (0.35)	No (20 μM)	130
1i	(CH <sub>2</sub> ) <sub>9</sub>	5 μM (0.79)	10 μM	20
1k	(CH <sub>2</sub> ) <sub>11</sub>	10 μM (0.80)	No (15 μM)	ND
1l	Ether (short)(CH <sub>2</sub> CH <sub>2</sub> O) <sub>3</sub> (CH <sub>2</sub> ) <sub>2</sub>	0.5 μM (0.42)	5 μM	3
1m	Ether (long)(CH <sub>2</sub> CH <sub>2</sub> O) <sub>4</sub> (CH <sub>2</sub> ) <sub>2</sub>	0.5 μM (0.25)	No (10 μM)	3
2a	H,H	No	No (5 μM)	ND
2b	Me,H	No	No (5 μM)	ND
2c	Me,Me	2 μM (0.40)	10 μM	ND
2d	SEM <sup>e</sup> ,H	No	No (5 μM)	ND
2e	SEM,SEM <sup>e</sup>	No	No (5 μM)	ND
2f	(CH <sub>2</sub> ) <sub>6</sub>	No	No (5 μM)	ND
2h	(CH <sub>2</sub> ) <sub>8</sub>	No	No (5 μM)	ND
2i	(CH <sub>2</sub> ) <sub>9</sub>	No	No (5 μM)	ND
2j	(CH <sub>2</sub> ) <sub>10</sub>	No	No (5 μM)	ND
2l	Ether (short)(CH <sub>2</sub> CH <sub>2</sub> O) <sub>3</sub> (CH <sub>2</sub> ) <sub>2</sub>	2 μM (0.81)	No (10 μM)	ND
2m	Ether (long) (CH <sub>2</sub> CH <sub>2</sub> O) <sub>4</sub> (CH <sub>2</sub> ) <sub>2</sub>	No	No (10 μM)	ND
3c	Me,Me	No	No (5 μM)	ND
3f	(CH <sub>2</sub> ) <sub>6</sub>	No	No (10 μM)	ND
3g	(CH <sub>2</sub> ) <sub>7</sub>	No	No (10 μM)	ND
3h	(CH <sub>2</sub> ) <sub>8</sub>	No	No (10 μM)	ND
3i	(CH <sub>2</sub> ) <sub>9</sub>	No	No (10 μM)	ND
3j	(CH <sub>2</sub> ) <sub>10</sub>	No	No (10 μM)	ND
6a	H,H	No	No (5 μM)	ND

(B) Effects of 7-Azaindolyl Indolylmaleimides (4 and 7) and Bis-7-Azaindolylmaleimides (5) on Self-Renewal<sup>a</sup>

Compound	R <sup>1</sup> , R <sup>2</sup>	SR <sup>b</sup>	Toxicity <sup>c</sup>	GSK-3β IC <sub>50</sub> <sup>d</sup>
4a	H,H	No	No (5 μM)	ND
4c	Me,Me	No	No (5 μM)	ND
4e	SEM,SEM <sup>e</sup>	No	No (5 μM)	ND
4h	(CH <sub>2</sub> ) <sub>8</sub>	5 μM (0.69)	No (15 μM)	ND
4i	(CH <sub>2</sub> ) <sub>9</sub>	5 μM (0.39)	No (10 μM)	ND
4j	(CH <sub>2</sub> ) <sub>10</sub>	No	No (5 μM)	ND
4l	Ether (short)(CH <sub>2</sub> CH <sub>2</sub> O) <sub>3</sub> (CH <sub>2</sub> ) <sub>2</sub>	5 μM (0.20)	No (10 μM)	16
4m	Ether (long) (CH <sub>2</sub> CH <sub>2</sub> O) <sub>4</sub> (CH <sub>2</sub> ) <sub>2</sub>	2 μM (0.65)	No (10 μM)	ND
5c	Me,Me	No	1 μM	ND
5i	(CH <sub>2</sub> ) <sub>9</sub>	5 μM (0.21)	No (10 μM)	ND
5m	Ether (long) (CH <sub>2</sub> CH <sub>2</sub> O) <sub>4</sub> (CH <sub>2</sub> ) <sub>2</sub>	10 μM (0.22)	No (10 μM)	90
7a	H,H	No	No (5 μM)	ND
7d	H,SEM <sup>e</sup>	No	No (5 μM)	ND

(C) Effects of Allyl-Substituted Bisindolylmaleimides on Self-Renewal<sup>a</sup>

Compound	R <sup>3</sup>	R <sup>4</sup>	SR <sup>b</sup>	Toxicity <sup>c</sup>	GSK-3β IC <sub>50</sub> <sup>d</sup>
1n	Si <sup>t</sup> BuPh <sub>2</sub>	H	No	No (10 μM)	ND
1o	H	H	0.5 μM (0.56)	5 μM	3
1p	CH <sub>2</sub> CH=CH <sub>2</sub>	H	5 μM (0.63)	No (10 μM)	30
8o	H	CH <sub>2</sub> Ph	No	No (5 μM)	ND
1q	CH <sub>2</sub> CH=CH <sub>2</sub>	H	10 μM (0.55)	No (15 μM)	ND



Table 1. continued

(C) Effects of Allyl-Substituted Bisindolylmaleimides on Self-Renewal<sup>a</sup>

Compound	R <sup>3</sup>	R <sup>4</sup>	SR <sup>b</sup>	Toxicity <sup>c</sup>	GSK-3 $\beta$ IC <sub>50</sub> <sup>d</sup>
1r	CH <sub>2</sub> CH=CH <sub>2</sub>	H	5 $\mu$ M (0.67)	No (15 $\mu$ M)	ND
1s	CH <sub>2</sub> OCH <sub>2</sub> CH=CH <sub>2</sub>	H	No	10 $\mu$ M	ND
1t	CH <sub>2</sub> CH <sub>2</sub> CH=CH <sub>2</sub>	H	No	10 $\mu$ M	ND

ND, not determined.

<sup>a</sup> A series of bisindolylmaleimides were tested for their ability to enhance self-renewal of mESCs as described in [Experimental Procedures](#).<sup>b</sup> The concentration at which formation of pure, highly self-renewing colonies emerged is given, along with the index of pure self-renewing colonies/total colonies at that concentration (in parentheses).<sup>c</sup> The concentrations at which the compounds induced cell death are reported. Where no cell death was observed, the concentration at which the compound was tested is indicated.<sup>d</sup> IC<sub>50</sub> values (in nM) were determined using recombinant protein and performed as part of the kinase profiling service provided by the Division of Signal Transduction Therapy, School of Life Sciences, University of Dundee to academic groups.<sup>e</sup> SEM, CH<sub>2</sub>O(CH<sub>2</sub>)<sub>2</sub>SiMe<sub>3</sub>.

of GSK-3 $\beta$  activity of 3 nM (Tables 1A and 1C). As shown in Figures 4A–4C, each of these molecules enhanced ESC self-renewal at concentrations of 0.5  $\mu$ M and above, compared to 5  $\mu$ M for **1i**. This functional activity correlated with their dose-response characteristics for GSK-3 inhibition, with doses of 0.5  $\mu$ M inhibiting  $\beta$ -catenin phosphorylation and enhancing TCF reporter activity 5- to 7-fold (Figures 4A–4C, panels ii and iii). Furthermore, following treatment with **1l**, **1m**, and **1o**, expression of Nanog and Oct4 were maintained, consistent with retention of pluripotency (Figures 4A–4C, panel iv; Figure S1). Molecules **2l**, **4m**, and **2c** were slightly more potent than **1i**, consistently promoting ESC self-renewal, inhibiting  $\beta$ -catenin phosphorylation, and enhancing TCF activity robustly at doses of 2  $\mu$ M and above (Figures 4D–4F).

We also characterized compounds **1p**, **4l**, and **5i**, which have potencies comparable to our lead molecule **1i**, and compounds **1h** and **5m**, which were less potent. These compounds enhanced self-renewal and inhibited  $\beta$ -catenin phosphorylation at concentrations of 5–10  $\mu$ M (Figure S2). In vitro kinase assays demonstrate that these molecules are the least effective at inhibiting GSK-3 $\beta$  activity (Table 1), with IC<sub>50</sub> values ranging from 16 nM (**4l**) to 130 nM (**1h**). Compounds that did not enhance self-renewal also did not inhibit  $\beta$ -catenin phosphorylation (not shown). We noted some significant differences between our results and a study by Kuo et al. (2003). This group also synthesized **1l**, **1m**, **4l**, and **5m**, and reported IC<sub>50</sub> values for GSK-3 inhibition of 22, 26, 17, and 48 nM, respectively (compared to our values of 3, 3, 16, and 90 nM). Why these are at variance is unclear. Surprisingly, whereas Kuo et al. report **5m** as their most specific GSK-3 inhibitor, with little or no inhibition of a panel of 50 kinases, in our hands this molecule is significantly less potent at enhancing ESC self-renewal than **1l** or **1m**.

#### Direct Effects of Bisindolylmaleimides on Other Signaling Pathways

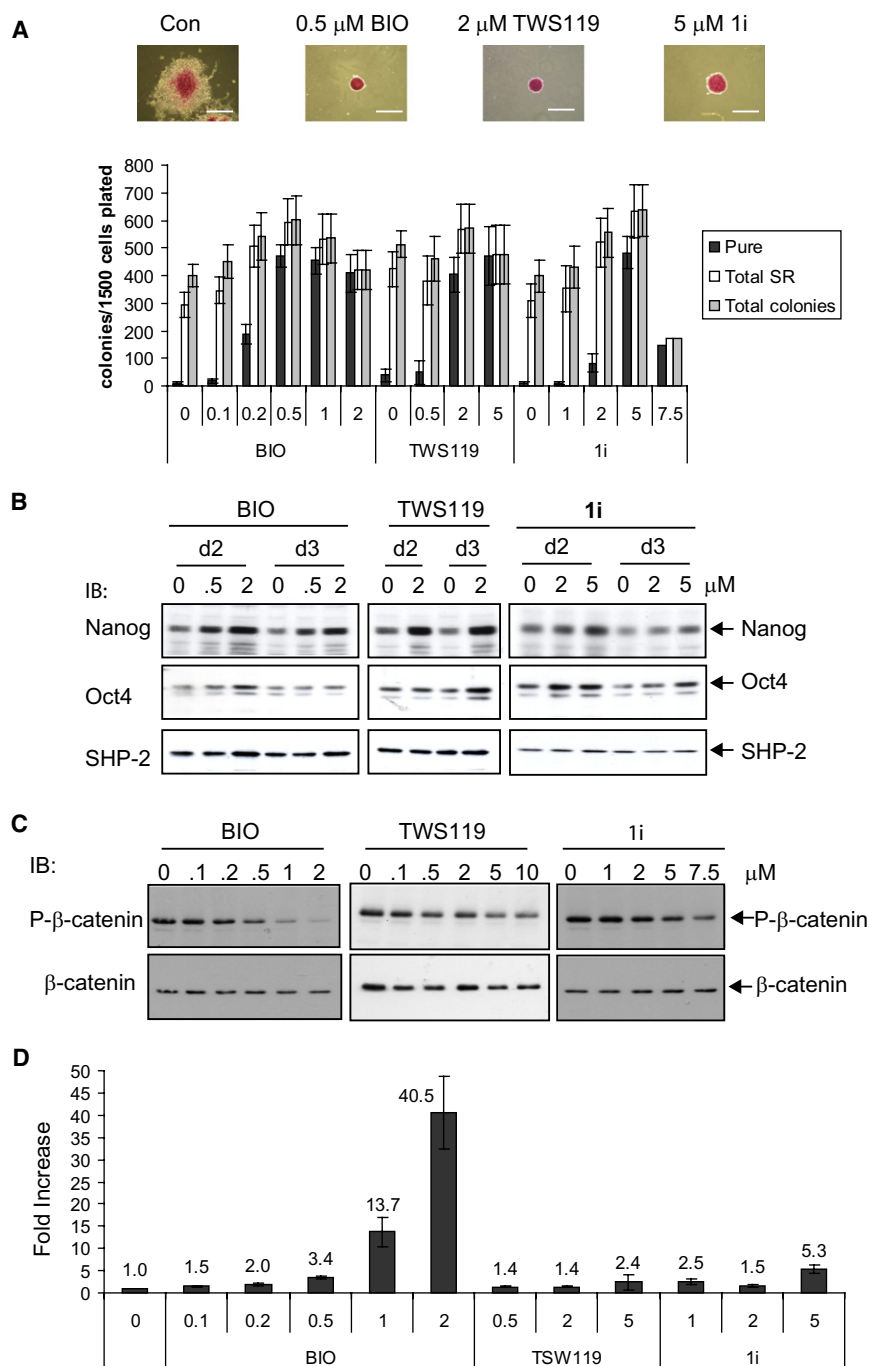
We wanted to assess the effects that BIO, TWS119, our lead compound (**1i**), and our three most potent compounds (**1l**, **1m**, and **1o**) had directly on other signaling pathways involved in ESC self-renewal. We examined the Stat3 pathway by measuring phosphorylation of Stat3 at the activating tyrosine Y705; PI3K/Akt signaling by analyzing effects on phosphorylation of ribosomal S6 protein at serines 235/236; and MAPK

signaling by analyzing phosphorylation of the TEY motif on Erk1 and 2. Following 30 min treatment with BIO (Figure 5A) or TWS119 (Figure 5B), there was a dramatic reduction in Erk1/2 phosphorylation as well as a decrease in Stat3 Y705 phosphorylation that were not observed with our most potent compounds (Figure 5). A similar reduction in Erk1/2 phosphorylation was observed following treatment of GSK-3 $\alpha/\beta$  double knockout (DKO) mESCs with BIO or TWS119 (Figure S3). S6 phosphorylation was only reduced by high concentrations of BIO and TWS119. Compounds **2l**, **4m**, **2c**, **1p**, **4l**, **5i**, **1h**, and **5m** were also assessed (Figure S4), and only high concentrations of **5i** led to a noticeable reduction of S6 phosphorylation. These results highlight that our compounds have little detectable effect on other downstream signaling pathways known to regulate self-renewal. This is in contrast to BIO and TWS119, which both had dramatic inhibitory effects on Erk1/2 signaling in wild-type and GSK-3 DKO ESCs.

#### Inhibition of GSK-3 in the Absence of LIF Does Not Sustain ESC Self-Renewal

Given the ability of a series of bisindolylmaleimides that act as GSK-3 inhibitors to enhance self-renewal in the presence of LIF and serum, and in view of earlier reports (Sato et al., 2004), we also tested whether BIO, **1i**, **1l**, **1m**, or **1o** could maintain self-renewal in the absence of LIF. After 5 days under these conditions, none of the compounds were able to maintain a robust population of pure compact self-renewing colonies (Figure 6A, panels i and ii). Alkaline phosphatase staining was only weakly enhanced in the presence of 0.5  $\mu$ M BIO, **1i**, **1l**, **1m**, or **1o** compared to untreated (Con) cells, whereas doses of 2  $\mu$ M **1l**, **1m**, and **1o** led to a decline in total colony number. Consistent with these effects, treatment with these inhibitors in the absence of LIF was not sufficient to maintain expression of Nanog or Oct4 (see Figure 6A, panel iii).

We set up similar assays to assess self-renewal using GSK-3 $\alpha/\beta$  DKO ESCs and compared these to conditionally targeted GSK-3 $\alpha^{(flx/flx)}$  cells (WT) (Doble et al., 2007). As seen in Figure 6B, panels i and ii, in the presence of LIF virtually 100% of the colonies generated by GSK-3 DKO cells were compact pure, highly self-renewing colonies, very similar to ESCs treated with our GSK-3 inhibitors. In contrast, in the absence of LIF there was a dramatic reduction in the number of pure self-renewing



**Figure 2. Compound 1i Treatment Enhances Self-Renewal of ESCs and Inhibits GSK-3-Mediated Signaling**

(A) mESCs were cultured in the presence of the indicated concentrations of compounds for 5 days and colonies were fixed and stained for alkaline phosphatase activity. The numbers of pure colonies (Pure), pure plus self-renewing colony types (Total SR), and total colony number (Total colonies) are shown. Data represent the mean  $\pm$  SEM of three independent experiments (except 7.5  $\mu$ M **1i**). Inset images show a typical self-renewing colony from untreated ESCs (Con) and typical pure highly self-renewing colonies formed in the presence of 0.5  $\mu$ M BIO, 2  $\mu$ M TWS119, or 5  $\mu$ M **1i**. The scale bars represent 400  $\mu$ m.

(B) ESCs were treated with the indicated concentrations of BIO, TWS119, or **1i** for 48 or 72 hr. Lysates were prepared and immunoblotting was carried out to detect expression of Nanog and Oct4. Blots were reprobed with anti-SHP-2 antibodies to assess loading.

(C) ESCs were treated with BIO, TWS119, or **1i** for 30 min. Immunoblotting was performed with an antibody detecting phosphorylated  $\beta$ -catenin. The same immunoblot was reprobed for total  $\beta$ -catenin to assess loading.

(D) TOPFlash luciferase reporter assay of ESCs treated for 24 hr with the indicated compounds. Data represent the mean  $\pm$  SEM of 3–12 independent experiments.

maleimides are capable of enhancing self-renewal in the presence of LIF and serum, but not in the absence of LIF. This property correlated strongly with the potency of GSK-3 inhibition while effects on other pathways influencing self-renewal were not observed. A role for GSK-3 in the regulation of ESC self-renewal is further supported by the demonstration that GSK-3 $\alpha/\beta$  null ESCs exhibit a similar enhancement of self-renewal.

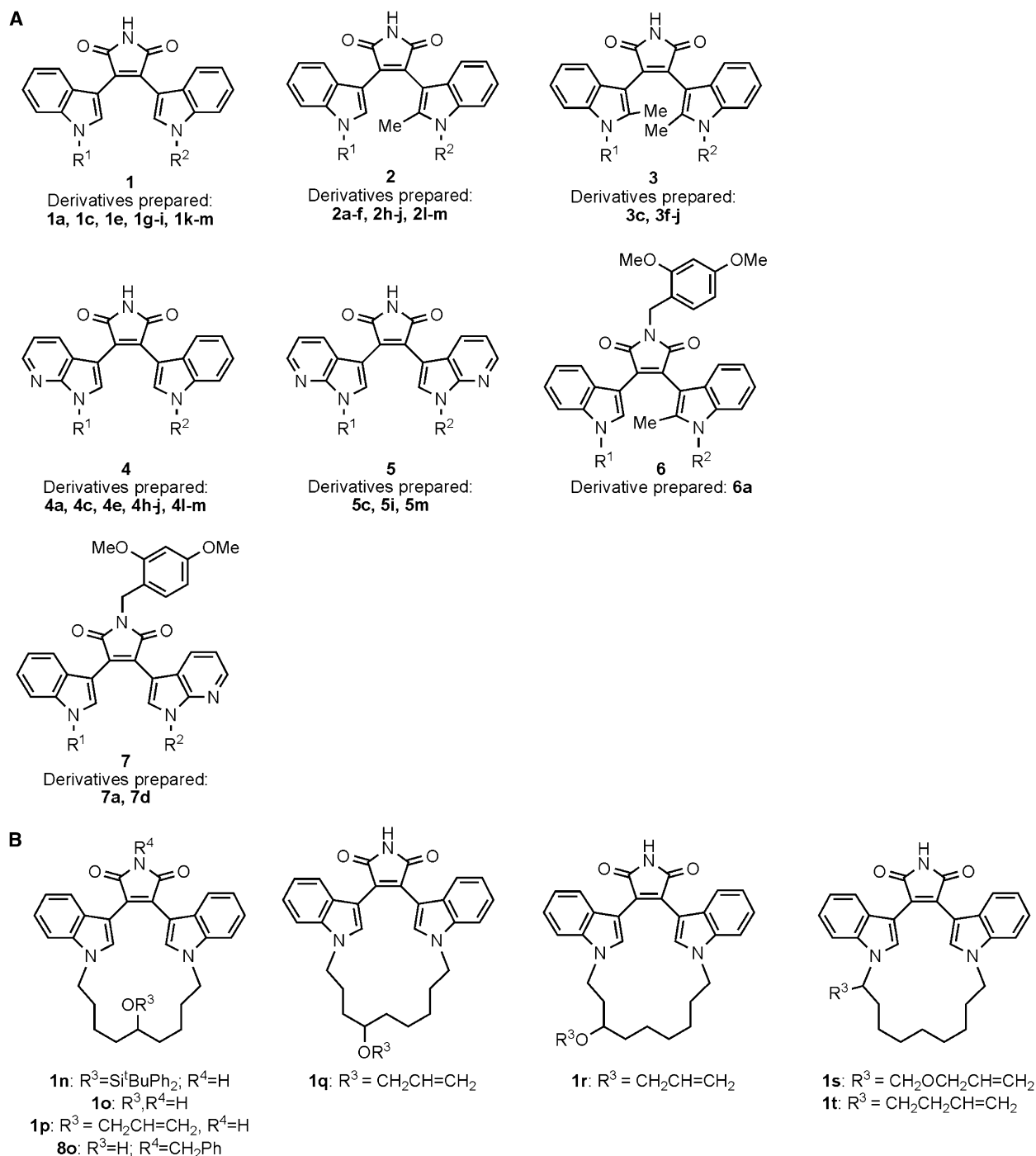
How GSK-3 functions to control ESC fate is not understood. GSK-3 has been reported to be involved in the regulation of a number of physiological processes including metabolism, cell survival, apoptosis, and translational control, with

colonies, accompanied by a decline in Nanog and Oct4 expression (Figure 6B, panel iii). Therefore, it appears that inhibition of GSK-3 cannot compensate for lack of LIF signaling to promote robust self-renewal, at least when the cells are plated at clonal density in the presence of serum.

## DISCUSSION

Using a chemical genetics-based approach, we present data supporting a role for GSK-3 in the regulation of self-renewal of mESCs. Our data clearly demonstrate that a series of bisindolyl-

more than 40 proteins reported to be substrates for GSK-3, among them metabolic and signaling proteins, structural proteins, and transcription factors (Jope and Johnson, 2004). Sato et al. first implicated GSK-3 in regulation of ESC self-renewal, their interpretation focusing on inhibition of GSK-3 as a surrogate activator of the Wnt pathway (Sato et al., 2004). However, inhibition of GSK-3 is likely to impact on numerous other pathways in ESCs. Of relevance here is the PI3K/Akt signaling pathway, activation of which by growth factors and cytokines leads to inhibition of GSK-3 activity, meaning GSK-3 inhibitors mimic activation of one of the PI3K/Akt effector



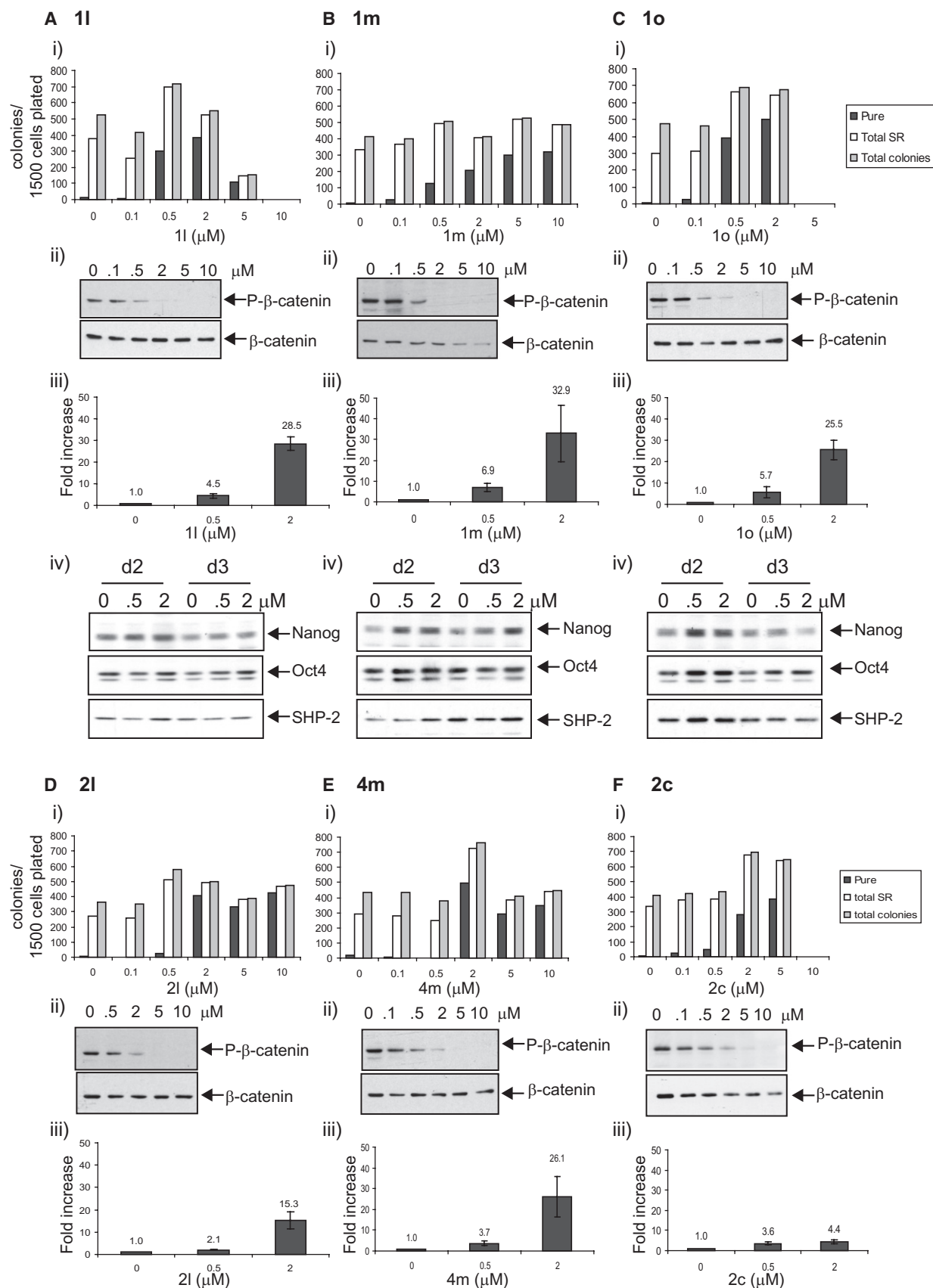
**Figure 3. The Compounds Used in This Study**

(A) The  $R^1$  and  $R^2$  substituents in the compounds are defined by the suffixes: **a:**  $R^1 = R^2 = \text{H}$ ; **b:**  $R^1 = \text{Me}$ ; **c:**  $R^1 = R^2 = \text{Me}$ ; **d:**  $R^1 = \text{CH}_2\text{O}(\text{CH}_2)_2\text{SiMe}_3$ ,  $R^2 = \text{H}$ ; **e:**  $R^1 = R^2 = \text{CH}_2\text{O}(\text{CH}_2)_2\text{SiMe}_3$ ; **f:**  $R^1, R^2 = -(\text{CH}_2)_6-$ ; **g:**  $R^1, R^2 = -(\text{CH}_2)_7-$ ; **h:**  $R^1, R^2 = -(\text{CH}_2)_8-$ ; **i:**  $R^1, R^2 = -(\text{CH}_2)_9-$ ; **j:**  $R^1, R^2 = -(\text{CH}_2)_{10}-$ ; **k:**  $R^1, R^2 = -(\text{CH}_2)_{11}-$ ; **l:**  $R^1, R^2 = -(\text{CH}_2\text{CH}_2\text{O})_3(\text{CH}_2)_2-$ ; **m:**  $R^1, R^2 = -(\text{CH}_2\text{CH}_2\text{O})_4(\text{CH}_2)_2-$ . Compounds with the suffixes **f–t** are macrocyclic analogs.

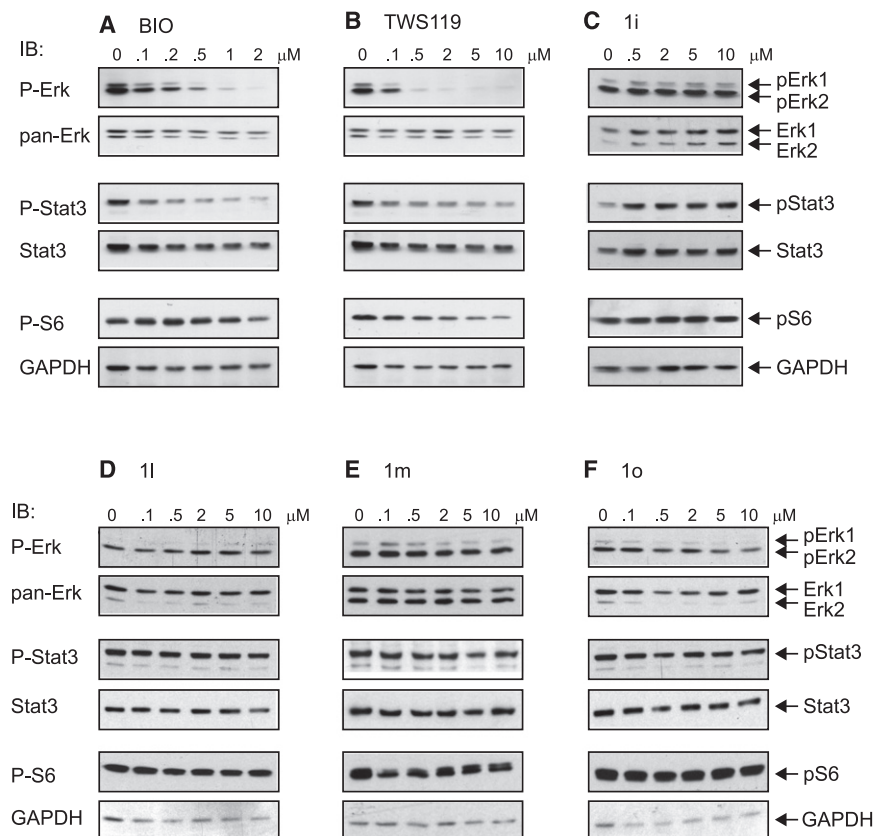
(B) The macrocyclic ligands with a functionalized tether that were used in this study.

pathways. Importantly, PI3K/Akt signaling has been shown to play a role in regulation of both murine (Paling et al., 2004; Watanabe et al., 2006) and human (Armstrong et al., 2006; Pyle et al., 2006) ESC self-renewal, whereas others have reported that

PI3Ks regulate ESC proliferation (Jirmanova et al., 2002; Sun et al., 1999; Takahashi et al., 2003). Although inhibition of PI3Ks increases GSK-3 activity, there is little evidence that PI3K/Akt signaling regulates GSK-3 localized within the







**Figure 5. Effect of Selected GSK-3 Inhibitors on Signaling Pathways in ESCs**

(A–F) ESCs were treated with the indicated compounds for 30 min. Immunoblotting was carried out on cell lysates extracted from the treated cells. The blots were probed with antibodies detecting phosphorylated forms of Erk1/2, Stat3, and S6 ribosomal protein. The same immunoblots in each case were reprobed for the corresponding total protein to evaluate loading, except for the pS6 blots, where GAPDH antibodies were used.

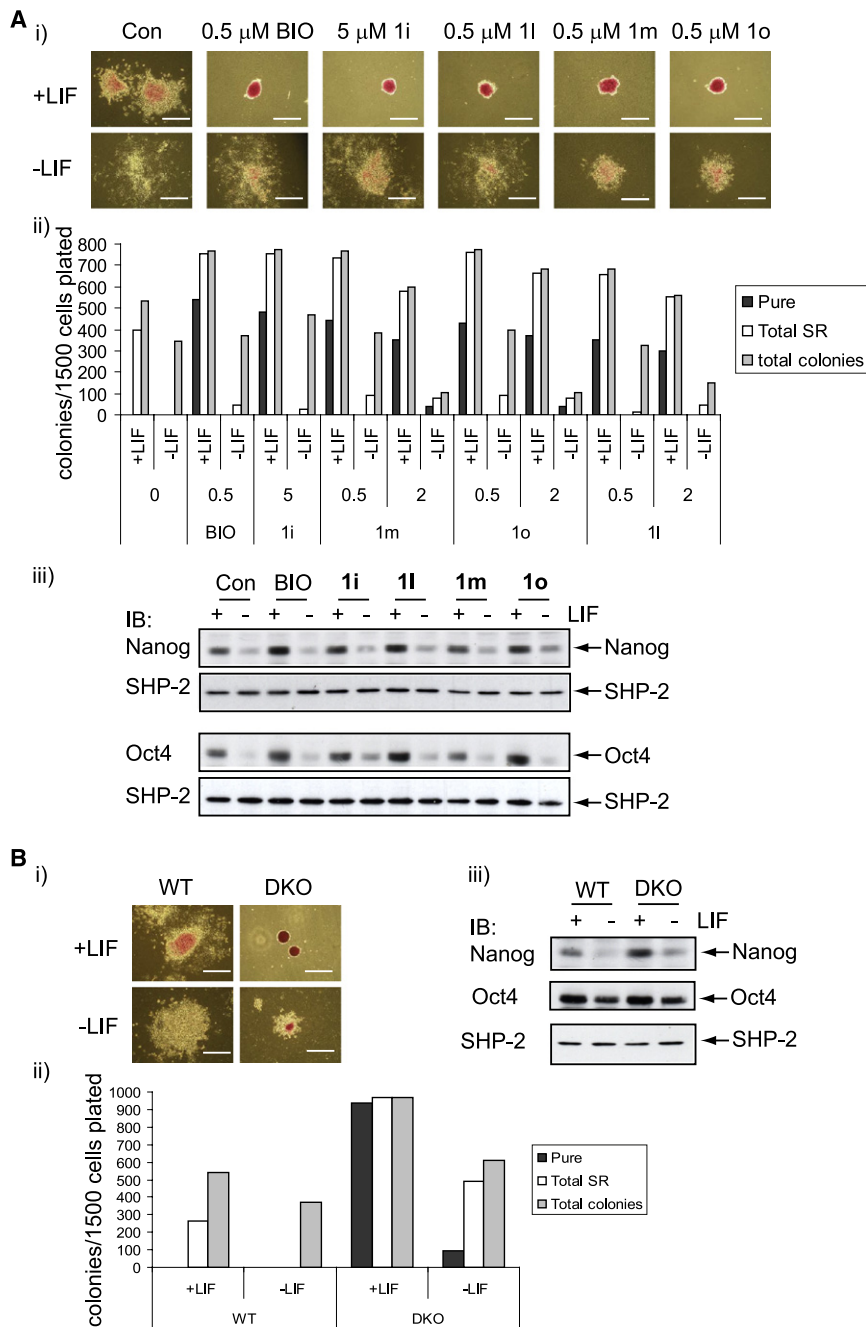
MAPK signaling and GSK-3 was sufficient to maintain ESC self-renewal in the absence of LIF, BMP4, and even Stat3, whereas inhibition of GSK-3 alone was not (Ying et al., 2008). They propose that under these defined conditions, GSK-3 inhibition is important for viability by maintaining metabolic activity and biosynthetic capacity. Our results demonstrate that in the presence of serum and LIF, conditions that already robustly support ESC metabolism and proliferation, inhibition of GSK-3 further enhances ESC self-renewal. We observed similar effects with GSK-3 $\alpha/\beta$  DKO ESCs, consistent with a previous report that GSK-3 $\alpha/\beta$  DKO ESCs are more resistant to differentiation (Doble

et al., 2007). This leads us to propose that in the presence of serum and LIF, rather than contributing to self-renewal primarily via maintenance of viability, as suggested by Ying et al., 2008, GSK-3 is involved in regulating self-renewal via alternate mechanisms (see above). Whereas addition of BIO to ESCs in the absence of LIF was reported to maintain self-renewal (Ogawa et al., 2006; Sato et al., 2004), our data, using a clonal functional assay, indicate that under these conditions, inhibition of GSK-3 alone is not sufficient to maintain self-renewal, consistent with results in serum-free conditions (Ying et al., 2008). Clearly further detailed studies are required to elucidate the exact mechanisms of action of GSK-3 in ESCs exposed to different environmental milieus.

Not only does our study provide robust evidence that GSK-3 is involved in regulation of ESC self-renewal but it also provides important insights into the structure–activity relationships of bisindolylmaleimides as inhibitors of GSK-3. Our previous work revealed that tether length played a critical role in constraining the conformation of bisindolylmaleimides, and this, in turn, controlled selectivity between alternative protein kinases; specifically, 1i was preorganized to function as a GSK-3 inhibitor

**Figure 4. Characterization of Compounds with Higher Potency in Enhancement of Self-Renewal**

ESCs were treated with compounds 1l (A), 1m (B), 1o (C), 2l (D), 4m (E), and 2c (F) and their effects on (i) self-renewal; (ii) and (iii) inhibition of GSK-3 activity and (iv) expression of Nanog and Oct4 were analyzed. (i) Colonies were fixed and stained for alkaline phosphatase activity and numbers of pure (Pure), self-renewing (Total SR), and total colonies (Total colonies) are shown. (ii) Immunoblotting to detect  $\beta$ -catenin phosphorylation was carried out as described for Figure 2C. (iii) TOPFlash reporter assays were performed on extracts from ESCs treated with the indicated concentration of compounds for 24 hr (bottom panel). Data represent the mean  $\pm$  SEM of three independent experiments. (iv) Immunoblotting to detect Nanog and Oct4 was carried out as described for Figure 2B.



**Figure 6. Inhibition of GSK-3 Does Not Sustain ESC Self-Renewal in the Absence of LIF**

ESCs were incubated with the compounds indicated (A) or wild-type (WT) and GSK-3 $\alpha/\beta$  double knockout (DKO) cells were cultured (B) in the presence of serum and the presence or absence of LIF for 5 days, after which colonies were fixed and stained for alkaline phosphatase activity. (i) Images show typical colonies formed from untreated ESCs (Con), and colonies formed in the presence of the indicated compounds (A) or colonies formed by WT and DKO ESCs (B) in the presence and absence of LIF. The scale bars represent 400  $\mu$ m. (ii) Alkaline phosphatase staining and the numbers of pure (Pure), self-renewing (Total SR), and total colonies (Total colonies) are shown. (iii) Immunoblotting to detect Nanog and Oct4 was carried out following 3 day treatments, as described for Figure 2B.

effect on Erk1/2 or Stat3 phosphorylation and in this respect are cleaner tools with which to probe GSK-3 function. This is further supported by the fact that in an antibody-based screen our lead molecule **1i** was found to have no significant effects on the phosphorylation of a wide variety of proteins.

Our main aim in initiating this study was to clarify the conflicting reports regarding the role of GSK-3 in regulation of ESC fate. Whereas TWS119 was reported to promote neuronal differentiation of EC and ES cells (Ding et al., 2003), BIO has been reported to enhance ESC self-renewal (Sato et al., 2004), promote proliferation of cardiomyocytes (Tseng et al., 2006), and preserve hemopoietic stem cell activity (Holmes et al., 2008). ESC cultures can be a mixture of self-renewing and differentiating cells, so if a signaling pathway has a role in both undifferentiated and differentiating cells, inhibition could have opposite effects depending on the context. Our finding that both TWS119 and BIO perturb additional path-

(Bartlett et al., 2005). Interestingly, our approach revealed that our three most potent molecules, **1l**, **1m**, and **1o**, are likely to adopt a ground-state conformation that is similar to that of **1i**. Furthermore, our comparison between reported inhibitors of GSK-3, namely BIO (Meijer et al., 2003; Sato et al., 2004) and TWS119 (Ding et al., 2003) and the bisindolylmaleimides characterized here, highlight a number of advantages of our molecules. Our analyses reveal that in addition to perturbing phosphorylation of  $\beta$ -catenin, treatment with BIO or TWS119 also reduced phosphorylation of Erk1 and 2 and Stat3, indicating that additional kinases may be targeted by these molecules. In contrast, our most potent compounds, **1l** and **1m**, have no detectable

ways prompts us to suggest that using only these molecules to probe GSK-3 action could lead to unreliable results. Given the recent report that inhibition of Erk-MAPKs and GSK-3 is sufficient to maintain self-renewal in the absence of exogenous factors (Ying et al., 2008) and our observation that BIO inhibits both GSK-3 and Erk1/2 signaling, it would be interesting to determine whether BIO maintains self-renewal in chemically defined media.

Overall, by using a chemical genetics-based approach, we have determined that GSK-3 plays an important role in regulating the ability of mESCs to self-renew. Our data, together with a recently published study (Ying et al., 2008), indicate that the

mechanism of action of GSK-3 may vary depending upon the ESC culture environment. Further studies are required to delineate the pathways involved in detail.

## SIGNIFICANCE

Embryonic stem cells (ESCs) and their differentiated progeny represent a renewable source of cells that are considered to be of wide utility in regenerative medicine, for cell-based therapies and tissue engineering, and as a source of differentiated cells for drug discovery, toxicity, and safety testing. However, in order for this potential to be realized, undifferentiated pluripotent ESCs need to be faithfully expanded to generate the large number of cells required for such purposes. To achieve this, it is imperative that the signals regulating ESC self-renewal, which underpins pluripotency, are understood in detail to ensure they are appropriately recapitulated during cell expansion. Previous investigations have suggested that inhibition of glycogen synthase kinase 3 (GSK-3) by small molecules can either promote ESC self-renewal, in the case of BIO, or facilitate neuronal differentiation, observed with TWS119. To interrogate the involvement of GSK-3 in regulation of ESC fate in detail, we adopted a chemical genetics-based approach. We demonstrate that in the presence of serum and LIF, inhibition of GSK-3 by a series of bisindolylmaleimides leads to robust enhancement of ESC self-renewal. The ability of these molecules to enhance ESC self-renewal is related to their potency of GSK-3 inhibition and, in contrast to BIO and TWS119, our most potent GSK-3 inhibitors do not have detectable effects on other pathways regulating self-renewal. This study further implicates GSK-3 in regulating mESC self-renewal and has provided cleaner tools to probe the function of GSK-3, applicable to many diverse cellular settings. We propose that by inhibiting GSK-3 activity, expansion of pluripotent ESCs will be facilitated, a significant benefit for applications requiring large numbers of undifferentiated cells. In future studies it will be interesting to determine whether GSK-3 plays a similar role in regulation of cell fate of induced pluripotent stem cells.

## EXPERIMENTAL PROCEDURES

### Culture of mESCs

The E14tg2a mESC line (Smith and Hooper, 1987) was cultured in Knock-out Dulbecco's modified Eagle's medium (Invitrogen) supplemented with  $10^3$  units/ml murine LIF (ESGRO; Chemicon) as previously described (Paling et al., 2004). GSK-3 DKO and GSK-3<sup>(lox/lox)</sup> cells (Doble et al., 2007) (a kind gift from Dr. J.R. Woodgett, Samuel Lunenfeld Research Institute, Toronto, ON, Canada and Dr. B. Doble, McMaster University, Hamilton, ON, Canada) were removed from feeders and maintained as described above.

### Alkaline Phosphatase Self-Renewal Assay

ESCs were plated at 1500 cells/well of a six-well tray in Glasgow modified Eagle's medium (GMEM) containing 10% (v/v) fetal bovine serum and  $10^3$  units/ml LIF. Following 4–6 hr, to permit adherence, compounds were added without media change. After 5 days, ESC colonies were stained to detect the presence of alkaline phosphatase (Paling et al., 2004). Colony images were visualized using an Olympus X151 inverted microscope and objective lens and captured using an Olympus Camedia C4040 digital camera.

### Short-Term Treatments and Preparation of Cell Lysates

Compounds were added to mESCs cultured in the presence of serum and LIF at the indicated concentrations for 30 min and lysates were prepared as previously described (Welham et al., 1994). Protein concentrations were determined using the Bio-Rad protein assay kit according to the manufacturer's directions. For preparation of cell extracts for antibody microarray screening,  $1 \times 10^6$  ESCs were lysed and protein samples (100  $\mu$ g) were analyzed by the Kinex antibody microarray service (KAM-1.1) performed by Kinexus (Vancouver, BC, Canada).

### Immunoblotting

Cell lysates (20  $\mu$ g) were separated by SDS-PAGE and transferred to nitrocellulose as described previously (Welham et al., 1994). Immunoblotting was carried out using primary antibodies at the following dilutions: 1:1,000 anti-GSK-3 $\beta$  (Cell Signaling Technology; CST 9315), anti- $\beta$ -catenin (CST 9562), anti-Oct4 (Santa Cruz Biotechnology; sc-9801), anti-Nanog (Abcam; Ab-21603); 1:2000 anti-Erk1 (sc-93), anti-GAPDH (sc-20357); 1:20,000 anti-phospho (Ser33/37/Thr41)  $\beta$ -catenin (CST 9561); 1:1,000 anti-phospho (Thr202/Tyr204) Erk1/2 (CST 9101), anti-phospho (Tyr705) Stat3 (CST 9131); 1:2,000 anti-phospho (Ser235/236) S6 ribosomal protein (CS 2211). Anti-rabbit antibodies conjugated to horseradish peroxidase (DAKO) were used at 1:10,000 and blots were developed using ECL or ECL Advance according to the manufacturer's directions (GE Healthcare). Blots were stripped and reprobed as previously described (Welham et al., 1994).

### ATP Bead Assay

KinaseBind  $\gamma$ -linked ATP agarose beads (Innova Bioscience) were prepared in wash buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 20 mM MgCl<sub>2</sub>, 1 mM DTT). mESC lysate (6 mg/ml) was incubated, with rotation, at 4°C with 400  $\mu$ l beads. After 3 hr the supernatant was removed (unbound) and the beads were washed six times. Beads were divided into seven aliquots, wash buffer was removed, and 50  $\mu$ l of the eluting compound (50  $\mu$ M BIO, **11**, or TWS119, or 10  $\mu$ M ATP) was added to the beads. After a 1 hr incubation at 4°C, the beads were pelleted and the supernatant was removed (eluted protein). Alternatively, mESC lysates were incubated at 4°C for 15 min with 5  $\mu$ M compound prior to addition of beads. Following a 3 hr incubation, unbound samples were removed, the beads were washed in wash buffer containing 5  $\mu$ M compound, and bound proteins were released by boiling the beads in 1 $\times$  sample buffer.

### GSK-3 Kinase Assay

GSK-3 specific activity was determined by measuring the transfer of  $^{32}$ P from [ $\gamma$ - $^{32}$ P]ATP to the GSK-3-specific peptide substrate GSM (Millipore; Ryves et al., 1998). To measure total GSK-3 activity, a 25  $\mu$ l reaction was used containing 12.5  $\mu$ l of ESC lysate (1 mg/ml) in the absence or presence of the indicated concentrations of compounds, 0.5 mg/ml GSM peptide substrate, 50 mM HEPES, 12.5 mM MgCl<sub>2</sub>, 2 mM DTT, 100  $\mu$ M ATP, and 2.5  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP (10  $\mu$ Ci/ $\mu$ l; 3000 Ci/mmol; GE Healthcare). After 15 min at room temperature, the assay was terminated by spotting 20  $\mu$ l onto P81 Whatman ion-exchange paper, which was washed four times (30 min each) in 0.6% phosphoric acid and dried, and bound radioactivity was quantified by scintillation counting.

### Luciferase Reporter Assay to Measure $\beta$ -Catenin-Mediated Transcriptional Activity

mESCs were cotransfected with 2  $\mu$ g TOPFlash (containing four consensus TCF binding sites upstream of the c-fos minimal promoter, driving expression of a firefly luciferase reporter) or FOPFlash (containing four mutant TCF binding sites) reporter constructs (kindly provided by C. Dani, CNRS, Nice, France) and 40 ng *Renilla* luciferase control vector pRL-TK (pRL-TK; Promega) using 20  $\mu$ l Lipofectamine 2000 (Invitrogen). After 24 hr, transfected cells were replated at  $2 \times 10^5$  cells in 2 ml GMEM/well of a 12-well tray and treated with compound as indicated. After a further 24 hr, cell extracts were prepared and firefly and *Renilla* luciferase activities were determined using the dual-luciferase reporter assay system according to the manufacturer's instructions (Promega). TOPFlash/FOPFlash firefly luciferase activities were normalized to those of cotransfected pRL-TK *Renilla* luciferase activity. Any FOPFlash activity was subtracted from TOPFlash activity and data were represented as fold increase above unstimulated control values.



## SUPPLEMENTAL DATA

Supplemental Data include four figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at [http://www.cell.com/chemistry-biology/supplemental/S1074-5521\(08\)00449-3](http://www.cell.com/chemistry-biology/supplemental/S1074-5521(08)00449-3).

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